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Johnsongrass (*Sorghum halepense*) Pollen Expresses ACCase Target-Site Resistance

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Three studies were conducted to develop pollen tests for the screening of acetyl coenzyme-A carboxylase (ACCase) target-site resistance in a biotype of johnsongrass. The assays were based on germination of johnsongrass pollen in media supplemented with clethodim. Two different methods were used to evaluate pollen germination—a visual assessment and a spectrophotometric assay. The response of pollen to the germination media was linear for 16 h. At 6 h after treatment, absorbance at 500 nm was nearly 0.5; consequently, 6 h was chosen to conduct the pollen assays using the spectrophotometer. Both assessment methods differentiated the susceptible (S) and resistant (R) biotypes. Pollen from the susceptible biotype of johnsongrass was strongly inhibited by increasing concentrations of clethodim, with a GR_{50} of 25.8 $\pm$ 0.6 (SE) μM and GR_{50} of 16.4 $\pm$ 1.7 (SE) μM clethodim by visual assessment and spectrophotometric assessment, respectively. Minimum R/S values were $>$ 3.9 by visual assessment and $>$ 6.1 by spectrophotometric assessment. ACCase target-site resistance is expressed in johnsongrass pollen.

Nomenclature: johnsongrass, *Sorghum halepense* (L.) Pers. SORHA.
Key words: Pollen assays.

Johnsongrass occurs in all major agricultural areas of the warm regions of the world and was listed by Holm et al. (1991) as one of the world's 10 worst weeds. Johnsongrass reduces crop yields (Horowitz 1973; McWorter and Hartwig 1972), and its pollen contaminates sorghum [*Sorghum bicolor* (L.) Moench] grown for seed (Rosenow and Clark 1969). In the early 1980s, the selective POST control of johnsongrass in soybean [*Glycine max* (L.) Merr.] first became possible with the registration of several herbicides now called graminicides (Burton 1997). The graminicides are divided into two chemically distinct herbicide classes, the aryloxyphenoxypropionates (AOPP) and the cyclohexanediones (CHD). Both of these herbicide classes act by inhibiting the enzyme acetyl coenzyme-A carboxylase (ACCase; EC 6.4.1.2) in susceptible species (Burton 1997). Acetyl coenzyme-A catalyzes the first committed step of fatty acid biosynthesis, which is the adenosine triphosphate–dependent carboxylation of acetyl-CoA to malonyl CoA (Incledon and Hall 1997). Grass species have a eukaryotic type ACCase in the chloroplast, which is sensitive to ACCase inhibitors, whereas most broadleaf species have a prokaryotic type of ACCase, which is not sensitive to ACCase inhibitors (Incledon and Hall 1997).

Resistance to the AOPP herbicides fluazifop-P and quizalofop and cross-resistance to the CHD herbicide sethoxydim was discovered in two populations of johnsongrass in Mississippi in 1991 (Smeda et al. 1997). Several CHD and AOPP herbicides were applied one or more times annually over several years in fields where resistant populations were detected. A second biotype was identified in Mississippi in 2000 that was resistant not only to the AOPP herbicides fluazifop-P and quizalofop and the CHD herbicide sethoxydim but also to the CHD herbicide clethodim (Burke et al. 2006d).

For plants, gene flow can involve pollen transport, also called pollen-mediated gene flow (Levin and Kerster 1974; Murray et al. 2002). The reproductive biology of species influence the degree of pollen-mediated gene flow between and within populations (Levin and Kerster 1974). Johnsongrass is a predominantly self-pollinating species with less than a 10% outcrossing rate (Dogget 1988). Even this limited amount of outcrossing could have implications for pollen movement of resistance. Consequently, understanding the biology and physiology of pollen could provide insight into the potential for movement of genes. Of special interest are genes imparting herbicide resistance.

Gene expression in the sporophytic generation overlaps with that of the gametophytic generation (Pedersen et al. 1987; Sari Gorla et al. 1986; Tanksley et al. 1981). As a consequence, many single, nuclear-encoded genes expressed in the sporophytic generation are also expressed in the gametophytic generation (Richter and Powles 1993) through which they are inherited. Richter and Powles (1993) showed that ACCase and acetolactate synthase (ALS) genes were expressed in pollen of rigid ryegrass (*Lolium rigidum* Gaud.). Furthermore, the enzymes encoded by these genes could be inhibited by herbicides. Richter and Powles (1993) could not detect resistance caused by something other than modification at the target site, which was attributed to a lack of expression of the enzymes conferring resistance. Thus, Letouze and Gasquez (2000) and Richter and Powles (1993) were able to detect target-site resistance in blackgrass (*Alopecurus myosuroides* Huds.) and rigid ryegrass, respectively. A bioassay involving pollen could be useful for detecting ACCase target-site resistance in johnsongrass, which would provide a better understanding of the potential for resistance movement through johnsongrass pollen. The objectives of these studies

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were to develop assays for detection of ACCase resistance in johnsongrass pollen to determine whether target site–based resistance is expressed in the gametophytic generation of johnsongrass.

Materials and Methods

Plant Material. Two populations of johnsongrass were used in this study: a susceptible population and a resistant population, which was highly resistant to clethodim (Burke et al. 2006d). Both populations are from Washington County, MS. The ACCase activity of the isolated enzyme from the resistant biotype in the presence of inhibitors was much higher than the susceptible biotype, indicating resistance due to an altered target site of action (Burke et al. 2006a).

Rhizomes of ACCase-resistant and -susceptible biotypes of johnsongrass were planted in 3.8-L round pots. The plants grown from these rhizomes were allowed to produce flowers. Biotypes were grown in separate glasshouses in Raleigh, NC, with a 12/12 h light/dark regime and fertilized once weekly with a 1 g/L solution of commercial plant fertilizer.[1] Average day/night temperatures were 32/18 C. Flowering commenced 6 to 8 wks after planting and continued indefinitely as long as old shoots and panicles were trimmed after flowering.

Pollen Germination and Clethodim Assays. Pollen was collected just before anthesis from plants that had been verified resistant or susceptible. To ensure consistent start times for each biotype and to prohibit cross-contamination, resistant pollen was harvested first, then susceptible pollen. To harvest pollen (and to reduce variation of pollen), three entire anthers from different flowers on three different plants were harvested, bulked, and immediately placed in the appropriate pollen germination media. Germination of pollen was performed in 1.5-ml microcentrifuge tubes containing 0.5 ml of an aqueous germination solution containing 0.3 M sucrose, 2.43 mM boric acid, and 3 mM calcium nitrate (Burke et al. 2006c).

Two methods were used to determine pollen germination in response to increasing rates of clethodim. The first method used a visual evaluation of percent pollen germination, whereas the second method used a spectrophotometer[2] to evaluate germination based on absorbance at 500 nm (Kappler and Kristen 1987). For the studies on pollen response to clethodim, a technical-grade clethodim stock solution (100 μM) was freshly prepared and diluted to give final concentrations of 10, 20, 40, 60, 80, and 100 μM when added to the germination solution. The solution that contained technical-grade clethodim was prepared by dissolving clethodim in acetone and adding deionized water to produce an acetone concentration of 33% (v/v). Clethodim concentrations were prepared in acetone, pollen germination media, and deionized water to maintain a consistent acetone content in each clethodim dilution. Acetone was used to equalize the total concentration of acetone in each clethodim dilution at 0.7%. In preliminary studies, 0.7% concentration of acetone did not affect johnsongrass pollen germination. Clethodim solutions were stored in the dark at 4 C until use.

The first study using visual evaluation of the percentage of pollen germination in response to increasing clethodim concentrations was arranged in a completely randomized design with three or four replications. Pollen was also germinated in a solution without clethodim and served as a nontreated check. After pollen had incubated 3 h (at 25 C) in the pollen germination media containing the different clethodim concentrations, the anthers were removed and the microcentrifuge tube was placed in a microcentrifuge for 1 min at 3,000 × g; 40 μl of the pollen germination media and pellet, containing both germinated and nongerminated pollen grains, were removed from the microcentrifuge tube using a micropipette and placed on a glass microscope slide. Pollen was scored as germinated if the pollen tube was at least half the size of the pollen grain (Richter and Powles 1993; Shivanna and Rangaswamy 1992). Six separate fields were selected from each slide, with each field containing 70 to 100 pollen grains. Pollen grains in each field were counted and scored as germinated or nongerminated. Percentage of germination was calculated for each field, and then, the six fields were averaged. Pollen was scored as germinated if the pollen tube was one-half the width of the pollen grain or greater. Using pollen collected separately, pollen viability was estimated using Alexander's stain (Alexander 1969). Before experiments were conducted, it was decided that only experiments where the control germination was greater than 60% of viable pollen as indicated by Alexander's stain would be scored (Richter and Powles 1993). However, both experiments had > 90% viability as indicated by Alexander's stain. The study, therefore, consisted of two experiments repeated in time.

To develop a spectrophotometric assay, it was necessary to first determine the incubation time necessary to detect pollen tube growth. To determine incubation time, a second study was conducted in which pollen was incubated in the pollen germination media (without clethodim) for 0.5, 1, 2, 4, 6, 8, or 16 h. After the specified time, the anthers were removed, and the microcentrifuge tube was placed in a microcentrifuge for 1 min at 3,000 × g. The pollen germination media was decanted, replaced with 0.25 ml of deionized water, and the pollen and pollen tubes homogenized with a pestle. The volume was brought to 1 ml, transferred to cuvette, and viewed in a spectrophotometer at 500 nm. At this wavelength, absorbance is a measurement of turbidity or the degree of opacity (Kappler and Kristen 1987). Pollen grains fixed in the pollen germination media by formaldehyde (final concentration 10 mM) were used as a zero-time control (Kappler and Kristen 1987). The absorbance values of the zero-time control were subtracted from those obtained in the experiments to account for the absorbance of the pollen grains without pollen tubes (Kappler and Kristen 1987). The study was conducted as a completely randomized design with three replications and was conducted twice.

In a third study, pollen germination in response to increasing clethodim concentration was evaluated spectrophotometrically (Kappler and Kristen 1987). Pollen was incubated in the pollen germination media for 6 h with increasing concentrations of clethodim including a clethodim-free control as described in study one. As with study one,

before experiments were conducted, it was decided that only experiments in which the control germination was greater than 60% of viable pollen, as indicated by Alexander's stain, would be scored (Richter and Powles 1993). Both experiments had > 90% viability. The study was conducted as a completely randomized design with three replications and was repeated in time.

Statistical Analysis. For the visual assessment and the spectrophotometric assay, pollen germination was expressed as a percentage of the treatment without clethodim. Data variance was visually inspected by plotting residuals to confirm homogeneity of variance before statistical analysis. Data were subjected to an ANOVA using the general linear models procedure in SAS (1998), and sums of squares were partitioned to evaluate the effect of trial repetition, herbicide rate, and johnsongrass biotype. The ANOVA indicated a significant biotype effect for the visual assessment and the spectrophotometric assay. Trial repetition and linear, quadratic, and higher-order polynomial effects of percentage of pollen germination over graminicide rates were tested by partitioning sums of squares (Draper and Smith 1981). Regression analysis was performed when the ANOVA indicated significant linear and nonlinear effects. Nonlinear models were used if the ANOVA indicated that higher-order polynomial effects of the percentage of pollen germination were more significant than linear or quadratic estimates. Estimation used the Gauss–Newton algorithm, a nonlinear least-squares technique (SAS 1998).

ANOVA indicated higher-order polynomial effects for percentage of pollen germination resulting from increasing herbicide rate for susceptible johnsongrass. Thus, percentage of pollen germination was modeled using the logistic function:

$$y = A + B \Big/ \left[1 + (x/GR_{50})^{d}\right] \quad [1]$$

where y is the response at dose x, A is the lower limit for y, B is the upper limit for y, d is the slope, and GR_{50} is the dose providing 50% injury or inhibition (Seefeldt et al. 1995). GR_{50} is most commonly referred to because it is the most accurate estimate of plant sensitivity to a herbicide (Seefeldt et al. 1995). When the logistic function was fit to the data, an approximate R^2 value was obtained by subtracting the ratio of the residual sum of squares to the corrected total sum of squares from one (Draper and Smith 1981). The logistic dose–response model could not be used on the data for the resistant biotype because the pollen from the resistant biotype did not respond to the concentrations of clethodim used in this study.

Results and Discussion

ANOVA indicated differences between the resistant and susceptible biotypes for each assessment method, and there was no trial main effect. Consequently, the response of johnsongrass pollen to increasing clethodim concentrations is presented by biotype averaged over experiments within each study. Pollen germination from the susceptible biotype of johnsongrass was strongly inhibited by increasing concentrations of clethodim (Figure 1), with a GR_{50} of 25.8 ± 0.6 (SE) μM clethodim. At 3 h after pollen immersion in pollen germination media, pollen from the resistant biotype, evaluated by visual assessment, germinated ≥ 80% at concentrations of 0 to 100 μM. Consequently, a logistic dose–response model did not fit the data.

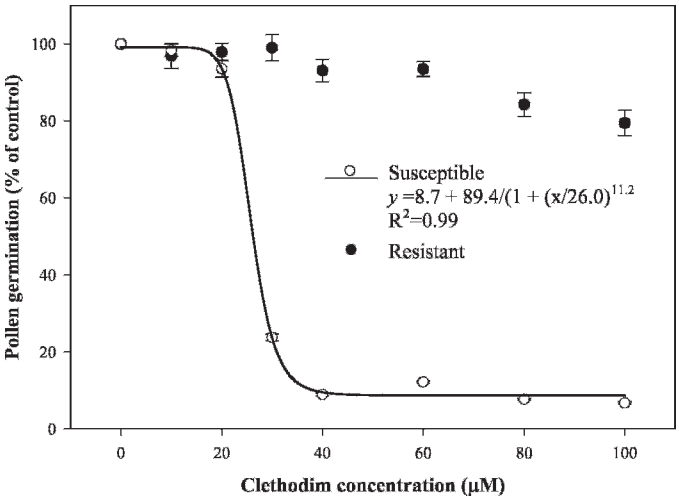

Figure 1. Effect of increasing concentrations of the acetyl coenzyme-A carboxylase–inhibiting herbicide clethodim on the germination of pollen from a susceptible and a resistant johnsongrass biotype as evaluated by visual assessment. Vertical bars represent standard error (n = 7).

The response of pollen to the germination media was linear for 16 h in the absence of clethodim regardless of biotype (Figure 2). At 6 h after treatment, absorbance at 500 nm was nearly 0.5. Consequently, 6 h was chosen to conduct the pollen assays using the spectrophotometer. An absorbance of 0.5 was selected as a balance between the level of absorbance and the time it takes to run the assay (6 h). When pollen germination was evaluated spectrophotometrically 6 h after treatment, the percentage of pollen tube growth inhibition for pollen from resistant johnsongrass was ≤ 13% at each clethodim concentration, resulting in relatively more turbid solutions than the susceptible biotype (Figure 3). As in the visual assessment, pollen from the susceptible biotype of johnsongrass was strongly inhibited by concentrations of clethodim > 10 μM (Figure 3), with a GR_{50} of 16.4 ± 1.7 (SE) μM clethodim. The spectrophotometric assay appears to be slightly more sensitive because it indicated pollen tube growth inhibition at a lower concentration of clethodim than that of the visual assessment for the susceptible biotype. A specific resistant/susceptible (R/S) ratio could not be computed because the highest clethodim rate did not reduce pollen germination of the resistant biotype by 50%. Minimum R/S values, calculated by taking the maximum clethodim concentration used (in μM) and dividing that by the GR_{50} (in μM) for the susceptible biotype, were > 3.9 by visual assessment and > 6.1 by spectrophotometric assessment. By comparison, R/S ratios using seedling assays, seedling whole-plant dose response, and rhizome whole-plant dose response were 18.7, 11.0, and 15.6 (Burke et al. 2006b, 2006d). The R/S ratio at the enzyme level was found to be 4.5

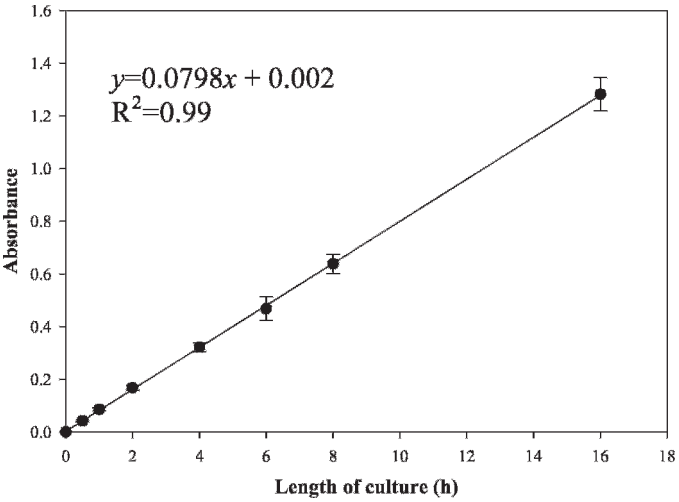

Figure 2. The effect of increasing duration of pollen culture on the optical density at 500 nm of homogenized pollen grains and tubes. Vertical bars represent standard error (n = 6).

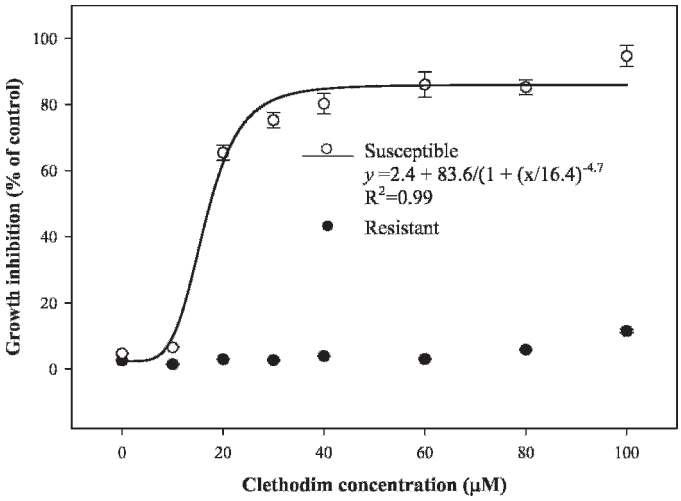

Figure 3. Effect of increasing concentrations of the acetyl coenzyme-A carboxylase–inhibiting herbicide clethodim on the germination of pollen from a susceptible and a resistant johnsongrass biotype expressed as growth inhibition as evaluated using a spectrophotometer at 500 nm. Vertical bars represent standard error (n = 6).

(Burke et al. 2006a). Both pollen assays were useful for distinguishing the resistant population from the susceptible population.

The resistant biotype of johnsongrass used in this study has a less-sensitive form of ACCase (Burke et al. 2006a). The resistant ACCase enzyme appears to be expressed in the pollen of this johnsongrass biotype. Others have noted that ACCase-resistant biotypes of other species that are resistant due to mechanisms other than target-site resistance are not identifiable using pollen assays (Letouze and Gasquez 2000; Richter and Powles 1993). It may be that the enzyme or enzymes that are responsible for metabolism-based resistance are not expressed in the pollen (Richter and Powles 1993). Therefore, these data lend support to the conclusion that this biotype of johnsongrass is resistant due to an insensitive target site.

Richter and Powles (1993) note that pollen screening will be useful in inheritance studies of target site–resistant traits. As pollen is haploid, heterozygous individuals will have intermediate levels of germination in response to a herbicide challenge (Richter and Powles 1993). Letouze and Gasquez (2000) were able to detect heterozygous individuals in populations of blackgrass as pollen from the heterozygous individuals germinated at intermediate levels to that of homozygous-resistant and -susceptible individuals. The population of resistant johnsongrass used in this study was treated with multiple applications of clethodim at 280 g/ha (two times the registered rate) (Anonymous 2005), and any injured plants were removed from the population before the development of the pollen assays. Reports indicate that the ACCase-resistance trait is partially dominant, and heterozygous individuals have intermediate levels of resistance at the whole-plant level (Smeda et al. 2000; Tal and Rubin 2004). The selection pressure may have removed heterozygous plants, and the plants used in this experiment may be homozygous resistant.

The pollen of johnsongrass and the genus *Sorghum* appear to be short-lived compared with other species. Burke et al. (2006c) found that pollen harvested just before anthesis was most germinable in vitro. Lansac et al. (1994) also found a complete loss of the ability of sorghum pollen to germinate after desiccation. Furthermore, Lansac et al. (1994) found greatly reduced seed set after 30 min of pollen desiccation. The short-lived nature of pollen from the genus *Sorghum* could have implications for the spread of the ACCase resistance found in the biotype that is the subject of this research. ACCase resistance provides an excellent marker to evaluate the mobility of johnsongrass pollen and the potential for movement of herbicide resistance in a mainly self-pollinating species. The pollen assays provide a good tool to determine resistance, an important part of studying the mobility of pollen (Levin and Kerster 1974). It may be that the short-lived nature of johnsongrass pollen limits the movement of resistance by pollen. Others have found that the contribution of pollen movement to resistance evolution and the spread of resistance in wild oat (*Avena fatua* L.) populations to be relatively small when compared with resistant seed production and dispersal from a resistant plant (Murray et al. 2002). Pollen assays coupled with a study on outcrossing using ACCase resistance as a marker would greatly increase the understanding of pollen-mediated gene flow in johnsongrass.

As pollen screening provides an indication of target site–based resistance, pollen assays appear to be useful tools for identifying the basis of resistance. These assays, though, are limited to species with germinable pollen. It is also necessary to determine the optimum pollen germination media before developing assays. Although pollen techniques are useful, a seedling assay is much simpler for extension personnel and others to perform. In conclusion, target site–based resistance to clethodim, an ACCase inhibitor, is expressed in the pollen of johnsongrass. Two assays were developed to detect resistance, one, a visual assessment using a microscope, and a second, an absorbance assessment using a spectrophotometer.

These assays will be useful in examining the pollen-mediated gene flow of ACCase resistance in populations of johnsongrass to gain a better understanding of resistance movement by pollen.

Sources of Materials

[1] Peters Professional 20–20–20, Scotts-Sierra Horticultural Products Co., 14111 Scottslawn Rd., Marysville, OH 43041.

[2] Perkin-Elmer UV/Vis Lambda 10 Spectrometer, 45 William Street, Wellesley, MA 02481-4078.

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